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Interaction of Mouse Adenovirus Type 1 Early Region 1A Protein

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We demonstrated functional associations between mouse adenovirus type 1 (MAV-1) early region 1A (E1A) protein and both the mouse retinoblastoma protein (pRb) and the mouse pRb-related protein, p107. Interactions between MAV-1 E1A and mouse pRb or mouse p107 proteins were examined in infected cell lysates using a mouse embryonic fibroblast cell line infected with wild-type and mutant MAV-1 viruses. Using a polyclonal antibody to MAV-1 E1A, exogenously added mouse pRb or mouse p107 was coimmunoprecipitated from wild-type-, dlE105 (CR1Δ)-, and dlE106 (CR3Δ)-infected cell lysates. No coimmunoprecipitation was seen with cell lysates from dlE102 (CR2Δ) or pmE109, a mutant virus that produces no detectable E1A protein due to an ATG to TTG point mutation in the initiator methionine. Introduction of mouse pRb into SAOS-2 cells resulted in a flat and enlarged cell phenotype, whereas cotransfection of mouse pRb and MAV-1 E1A resulted in a significant reduction of flat cells, presumably due to E1A binding pRb. CR1Δ and CR2Δ E1A proteins were less effective at reducing the number of flat, enlarged cells induced by pRb expression than were the CR3Δ or wild-type E1A proteins. The reduced ability of these mutants to inactivate pRb relative to wild-type E1A correlated with their reduced ability to bind pRb in the *in vitro* coimmunoprecipitation experiments. As a measure of p107/MAV-1 E1A complex formation in MAV-1-infected cells, we used mobility shift assays to examine cell extracts for the presence of p107-containing E2F protein–DNA complexes. Mock-, dlE102-, and pmE109-infected cell extracts formed a p107-containing complex, whereas wild-type-infected cell extracts did not. Thus the formation of a p107–E2F complex in wild-type- or these mutant-infected extracts inversely correlated with the presence of E1A–p107 complexes identified in the *in vitro* coimmunoprecipitation experiments. This is consistent with E1A–p107 complexes forming in wild-type MAV-1-infected cells.

INTRODUCTION

Viral and host cell protein interactions play an important role in the pathogenesis of viral infections. Interactions that occur during human adenovirus (hAd) infections of cultured cells have been identified. Early region 1A (E1A) proteins of hAds are multifunctional and associate with a number of host proteins. Many of these cellular proteins are involved in cell cycle regulation. It is hypothesized that host protein–E1A interactions are important in altering the state of infected cells, causing them to enter the S phase of the cell cycle, thereby becoming more permissive for viral DNA synthesis (Whyte *et al.,* 1988). E1A proteins function to regulate viral gene expression (Berk *et al.,* 1979; Jones and Shenk, 1979), activate transcription of both viral and cellular genes (Berk *et al.,* 1979; Jones and Shenk, 1979; Nevins, 1982; Gaynor *et al.,* 1984; Simon *et al.,* 1987), and repress viral and cellular transcriptional enhancers (Borelli *et al.,* 1984; Hen *et al.,* 1985; Velcich and Ziff, 1985). E1A proteins also transform nonpermissive cells (Ruley, 1983) and induce cell cycle progression by induction of cellular DNA synthesis and mitosis in growth-arrested primary rodent cells (Shimojo and Yamashita, 1968; Younghusband *et al.,* 1979; Braithwaite *et al.,* 1983; Spindler *et al.,* 1985).

Mutational analysis of the hAd E1A gene has allowed correlation of many of the biological activities attributed to E1A with three specific regions of the protein that are highly conserved among adenoviruses. The ability to transactivate other genes resides within conserved region 3 (CR3) of the 289-amino-acid (aa) E1A protein (Svensson and Akusjärvi, 1984). Conserved regions 1 (CR1) and 2 (CR2) and the N-terminus are required for transformation of rodent cells (Stein *et al.,* 1990; reviewed in Boulanger and Blair, 1991, and Shenk and Flint, 1991; Corbeil and Branton, 1994). Induction of DNA synthesis is dependent on CR1 and CR2, whereas induction of mitosis and further cellular proliferation require only CR2 (Zerler *et al.,* 1987; Moran and Zerler, 1988; Howe *et al.,* 1990; Stein *et al.,* 1990; Howe and Bayley, 1992). Host proteins retinoblastoma protein (pRb), pRb-related protein (p107), p130, and cyclin A have been demonstrated to associate with CR1 and CR2 of hAd E1A (Egan *et al.,*

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1988; Jelsma *et al.,* 1989; Whyte *et al.,* 1989; Giordano *et al.,* 1991; Li *et al.,* 1993). Another cellular protein, p300, also associates with E1A, requiring CR1 and the nonconserved N-terminus of E1A for this association (Wang *et al.,* 1993). The E1A regions required for association with these cellular proteins are also essential for the various E1A functions displayed during an infection of cultured cells. It is likely that E1A exerts its activity through these cellular protein interactions. Hypotheses regarding the functional importance of E1A–host cell protein interactions during an infection have been proposed (Whyte *et al.,* 1988), but have not been tested in the natural host. The species specificity of adenoviruses limits the study of hAds to cell culture or nonpermissive animal hosts. Study of mouse adenovirus type 1 (MAV-1) enables us to correlate viral and host protein interactions with specific aspects of pathogenesis in the natural host.

Initial characterization of MAV-1 E1 revealed a single major E1A transcript which overlaps the early region 1B (E1B) transcripts (Ball *et al.,* 1989). Thus MAV-1 E1 differs from hAd E1, which has two major E1A transcripts that do not overlap E1B. We demonstrate here that the single predicted MAV-1 E1A protein of 200 aa is made in infected cells. The MAV-1 E1A protein has approximately 40% amino acid similarity to the 289-aa E1A protein from hAd4, hAd5, and hAd7 within the three conserved regions (Ball *et al.,* 1988). Of the three conserved regions, MAV-1 CR2 (aa 112–128) has the highest degree of similarity to hAd CR2, with more than 50% of the residues being identical or similar (Ball *et al.,* 1988). In particular it possesses the pRb binding motif (D)-L-X-C-X-E found in most hAd E1A proteins, the SV40 large T antigen, and E7 protein of human papilloma virus 16. Because this region has been shown to bind the cellular proteins pRb and p107 (Egan *et al.,* 1988; Whyte *et al.,* 1989), we investigated whether MAV-1 E1A associates with mouse cellular pRb and p107. We used biochemical and functional assays to demonstrate that these interactions occur during a MAV-1 infection.

MATERIALS AND METHODS

Cells and viruses

Mouse 3T6 fibroblast cells and mouse L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated calf serum. The human osteogenic sarcoma SAOS-2 cells obtained from the American Type Culture Collection were maintained in DMEM supplemented with 15% heat-inactivated fetal bovine serum.

Mouse 37.1 cells

pZ112.F is a plasmid containing MAV-1 E1A cDNA sequence in the opposite orientation from pZ112 (Ball *et al.,* 1989). pZ112.F was linearized with *Bst*BI (nt 2984 in MAV-1 sequence) and filled in using Klenow to produce blunt ends. It was then digested with *Hin*dIII (5′ of E1A sequence), and the MAV-1 E1A-containing fragment was inserted into *Hin*dIII–*Eco*RV-digested pcDNAI/Amp (Invitrogen) such that the E1A sequence was under the control of the CMV promoter. This plasmid, pCME1A, was then digested with *Hin*dIII and *Xba*I and ligated with similarly digested pJ5Ω (Morgenstern and Land, 1990). In the resulting plasmid, pE1AΩ, E1A is under the control of the MMTV promoter. Mouse 3T6 cells plated in 35-mm wells were cotransfected with 1 μg pE1AΩ and 0.1 μg pcDneo (Chen and Okayama, 1987) using Lipofectamine (GIBCO BRL), following the manufacturer's standard protocol. Two days after transfection, 400 μg/ml G418 was added for selection. Two weeks after transfection, isolated colonies were picked and expanded. Genomic DNAs were prepared from the cells (Laird *et al.,* 1991) and tested for the presence of E1A sequences by the polymerase chain reaction (PCR) using oligonucleotides CR1 and MAVL883 as primers (Table 1). Positive cell lines were then tested by Western blot analysis for expression of E1A protein after induction with 10^{-6}–10^{-7} *M* dexamethasone. Six lines were isolated that expressed E1A (data not shown), and the line designated 37.1 was used in the studies presented here. 37.1 cells were maintained in DMEM containing 5% heat-inactivated calf serum and 200 μg/ml G418. For virus propagation, cells were passaged one time in the absence of G418, and 10^{-5} *M* dexamethasone was added 5–24 hr prior to infection. Dexamethasone was included in the agarose overlay of plaque assays.

MAV-1 mutant virus construction

Mutations were introduced into plasmids containing genomic or cDNA sequences of MAV-1 by site-directed mutagenesis using oligonucleotides indicated in Table 1. Oligonucleotides were synthesized by Operon Technologies or the Molecular Genetics Instrumentation Facility, University of Georgia. Mutagenesis was carried out on single-strand templates using the appropriate primer and the Amersham oligonucleotide-directed *in vitro* mutagenesis system according to the manufacturer's instructions. Templates and the resulting plasmids with E1A mutations are indicated in Table 2. Mutations were verified by restriction digest and/or DNA sequencing.

pmE301 virus was isolated after cotransfection by the calcium phosphate precipitation method (Gorman *et al.,* 1983) of mouse 3T6 cells with pMUT2 linearized with *Hin*dIII (Table 2) and MAV-1 DNA–protein complex (isolated as in Dunsworth-Browne *et al.,* 1980). A single mutant virus plaque was identified by restriction digest of Hirt DNA (Hirt, 1967), plaque purified three times, and used to produce a virus stock. pmE301 virus has a single point mutation in the first intron of early region 3 which eliminates the *Eco*RI site while maintaining the amino

TABLE 1

Oligonucleotides Used in This Work

Name	Sequence	Use
E3R1	5′ TCTCGAGAGTTCCCCCCTAC 3′	Eliminate *Eco*RI site at 79.4 m.u.
ATGoligo	5′ CTTTTGACTCATTGTCGCG 3′	Mutate E1A initiator ATG to TTG.
CR1	5′ GTATGTCGTACGCCAGATTCTACTACTGCC 3′	Delete E1A aa 35–78.
CR2	5′ GTGGAGAGTTTTGAGATTGAGGTGTTCCCG 3′	Delete E1A aa 111–129.
CR3g	5′ ATTGAGGTGTTCCCGGGTCTGACTGGTAAG 3′	Delete E1A aa 135–154 in genomic clones.
CR3c	5′ ATTGAGGTGTTCCCGGGTCTGACTGATCTG 3′	Delete E1A aa 135–154 in cDNA clones.
MAVL170	5′ GGTTTTTTACTTTGCGGAGC 3′	Left side PCR primer for E1A mutant screening.
MAVL255A	5′ GGTGCGATTTTTCGACTTTTGACTCAA 3′	Left side PCR primer for E1A mutant screening.
MAVL255T	5′ GGTGCGATTTTTCGACTTTTGACTCAT 3′	Left side PCR primer for E1A mutant screening.
MAVL883	5′ GCAGACTCATCAGGAACTTC 3′	Right side PCR primer for E1A mutant screening.
MAVL1134	5′ GGTAACATATTGCAGCCTAC 3′	Right side PCR primer for E1A mutant screening.
RoxL98	5′ CATGCCGCCCAAAGCCCCGCGCAGAGCCGC 3′	Linker for pCiteRb construction.
RoxL98c	5′ GGCTCTGCGCGGGGCTTTGGGCGG 3′	Linker for pCiteRb construction.

acid sequence of pVIII (Raviprakash *et al.,* 1989; Beard *et al.,* 1990). pmE301 behaves like wild-type MAV-1 in all characteristics assayed to date (Spindler, unpublished), and is referred to as wild-type MAV-1 throughout this work.

To construct viruses with mutations in E1A, we used a method similar to one developed for making MAV-1 E3 mutants (Beard and Spindler, 1996). pmE301 DNA–protein complex was digested with *Eco*RI and partially filled in using Klenow enzyme in the presence of dATP, and the Klenow reaction was terminated by the addition of EDTA to 25 m*M.* Plasmids containing mutated genomic MAV-1 sequence (pTTG, pCR1g, pCR2g, or pCR3g, see Table 2) were digested with *Hin*dIII and *Xba*I, releasing the MAV-1 insert. The plasmid DNAs were then treated with *Eco*RI methylase to protect them from digestion with *Eco*RI present in the pmE301 DNA–protein complex. The plasmid DNAs were ethanol precipitated, mixed with the partially filled-in pmE301 DNA–protein complex, and cotransfected into mouse 37.1 cells by the calcium phosphate precipitation method (Gorman *et al.,* 1983). Five micrograms of DNA–protein complex was mixed with 4 μg plasmid DNA, the total DNA was adjusted to 20 μg with herring sperm DNA, and the calcium phosphate precipitate was divided between two 60-mm plates of cells. Twenty-four hours after transfection the plates were overlaid with DMEM containing 2% heat-inactivated calf serum, 10^{-5} *M* dexamethasone, and 0.6% agarose.

Plaques were analyzed for the presence of mutant virus as follows. The plaques were picked into 0.5–1 ml of phosphate-buffered saline (PBS). An aliquot (0.125–0.5 ml) of this was mixed with 0.1 volume of 4.5% NP-40/4.5% Tween 20. Proteinase K (250 μg/ml) was added, and the samples were digested at 55° for 60 min. Samples were incubated at 95° for 10 min to inactivate the proteinase K, ethanol precipitated, and resuspended in 10 μl water. One to five microliters was used as template for PCR amplification. To identify deletion mutants, oligonucleotide primers flanking the mutation were used: MAVL170 and MAVL1134 primers for the CR1 and CR3 deletions, and CR1 and MAVL883 primers for the CR2 deletion (Table 1). Plaques arising from the cotransfection of pTTG were analyzed by differential PCR utilizing MAVL255A or MAVL255T and MAVL883 primers (Table

TABLE 2

Templates and Resulting Mutagenized MAV-1 Plasmids and Viruses

Template[a]	Mutagenic oligonucleotide[b]	Mutagenized plasmid	Mutant virus
pBHC2	E3R1	pMUT2	pmE301
pZ112	CR1	cΔCR1 (E1A cDNA)	
pZ112	CR2	cΔCR2 (″)	
pZ112	CR3c	cΔCR3 (″)	
pMXD	ATGoligo	pTTG (E1A genomic DNA)	pmE108, pmE109
pMXD	CR1	pCR1g (″)	dlE104, dlE105
pMXD	CR2	pCR2g (″)	dlE102, dlE103
pMXD	CR3g	pCR3g (″)	dlE106, dlE107

[a] pBHC2, MAV-1 *Hin*dIII-C fragment cloned into pBS$^+$ (Stratagene); pZ112 (Ball *et al.,* 1989); pMXD (Ball *et al.,* 1988).
[b] See Table 1.

1). The PCR reactions for the differential screening contained 0.5 m*M* Mg^{2+}, and the annealing temperature of 52° was used to discriminate between priming of the wild-type (MAVL255A) or mutant (MAVL255T) oligonucleotide from wild-type or mutant plasmid template controls. Plaques containing desired mutations were plaque-purified three times and virus stocks were prepared on 37.1 cells. To verify the purity of the stocks and the presence of the mutations, viral DNA samples were prepared for PCR analysis from 100 μl of tissue culture supernatant by boiling 5 min. The samples were then mixed briefly with 10 μl StrataClean resin (Stratagene) and pelleted, and the supernatant containing DNA was transferred to a fresh tube. One microliter of the supernatant was used in PCR fmol sequencing (Promega) and all mutant viruses had the expected mutations. The viruses were named as indicated in Table 2.

Plasmids used for *in vitro* production of mouse cellular proteins

pCiteRb contains the full length mouse pRb cDNA downstream of a cap-independent translation enhancer sequence to increase the *in vitro* translation efficiency in rabbit reticulocyte lysates. To construct pCiteRb, a fragment containing the mouse pRb cDNA was gel isolated from *Sac*II–*Xmn*I-digested pBSK+115Rox, which is a derivative of pJ3-115 (Bernards *et al.,* 1989), provided by J. Horowitz. The isolated fragment was digested with *Bam*HI and the resulting *Bam*HI–*Sac*II fragment was gel purified, mixed with the RoxL98 and RoxL98c linkers (Table 1), and ligated to *Bam*HI–*Nco*I-digested pCite-2b (Novagen), resulting in pCiteRb. Mouse p107 cDNA under the control of the T7 promoter in pmp107 was used for *in vitro* transcription and translation and was provided by E. Harlow (Zhu *et al.,* 1993).

The mouse pRb expression plasmid used in the SAOS-2 transfection experiments, pmRb-SVE, was constructed as follows from a mouse pRb cDNA containing plasmid, pT7115.Rox, a derivative of pJ3-115 obtained from R. Bernards (Bernards *et al.,* 1989). pSVE was obtained by removing hAd E1A sequences from 13S-SVE (provided by P. Hinds). pSVE [a variant of pJ3Ω (Morgenstern and Land, 1990)], whose backbone is the large *Pvu*II fragment of pBluescript (SK^+), was linearized with *Kpn*I, and the ends were blunted using T4 DNA polymerase. This was ligated to the *Pvu*II fragment of pT7115.Rox containing the mouse pRb cDNA, resulting in pRox-SVE. The large *Eco*RI–*Pst*I fragment of pRox-SVE was isolated and ligated to the *Eco*RI–*Pst*I fragment of pT7115.Rox, resulting in pmRb-SVE which contains the entire mouse pRb cDNA downstream of the SV40 promoter.

Construction of MAV-1 E1A expression plasmids

For the SAOS-2 transfection experiments, MAV-1 E1A wild-type and mutant cDNAs were subcloned into pcDNAI/Amp at the *Eco*RV site. The wild-type MAV-1 E1A expression construct, pCME1A, is described above. pcΔCR1, pcΔCR2, and pcΔCR3 are derivatives of pZ112 (Ball *et al.,* 1989) in which nt 382–515, 610–668, and 682–742 of E1A are deleted, respectively. To construct these plasmids, deletions were introduced into pZ112 by site-directed mutagenesis as described above using oligonucleotides CR1, CR2, and CR3c (Table 1), resulting in pcΔCR1, pcΔCR2, and pcΔCR3, respectively. pCME1A, pcΔCR1, pcΔCR2, and pcΔCR3 were digested with *Bst*BI, filled in using Klenow, and digested with *Eco*RI. The cDNA-containing fragments were gel isolated and ligated to an *Eco*RV–*Eco*RI-digested pcDNAI/Amp vector resulting in pCMV-CR1Δ, pCMV-CR2Δ, and pCMV-CR3Δ. The MAV-1 E1A initiator mutant construct, pCMV-TTG, was made by digesting pTTG with *Bst*BI, filling in using Klenow, then digesting with *Hin*dIII. The 2984-bp *Bst*BI–*Hin*dIII fragment of pTTG was gel isolated and ligated to a *Hin*dIII–*Eco*RV-digested pcDNAI/Amp, resulting in pCMV-TTG.

Antisera

The monoclonal antiserum, XZ104, raised against a human pRb polypeptide corresponding to amino acids 387–928, was provided by E. Harlow (Hu *et al.,* 1991). The mouse pRb polyclonal antiserum was made against a GST–mouse pRb fusion protein expressed from pGEX-1NMuRB, a plasmid containing the N-terminal 386 aa of mouse pRb. Antisera and plasmid were both provided by J. Horowitz (Sterner *et al.,* 1995). We produced additional antiserum by inducing fusion protein expression in *Escherichia coli,* collecting the fusion protein on glutathione agarose beads, and using it to immunize rabbits (University of Georgia Animal Resources). SD9 is a monoclonal antiserum raised against human p107 which cross-reacts with mouse p107 and it was kindly provided by E. Harlow (Dyson *et al.,* 1993).

A polyclonal antiserum to MAV-1 E1A was produced as follows. The MAV-1 E1A cDNA sequence from nt 360 to 3260 of Z112 (Ball *et al.,* 1989) was inserted as a *Bam*HI–*Hin*dIII fragment into *Bam*HI–*Hin*dIII-digested pATH2 (Koerner *et al.,* 1991), producing an *E. coli trpE*–MAV-1 E1A fusion protein expression plasmid, pTE1A29. The fusion protein expressed by pTE1A29, consisting of MAV-1 E1A aa 27–200, was overproduced in *E. coli,* isolated from polyacrylamide gels, and used to immunize rabbits (Bethyl Laboratories, Inc.). Antisera from two rabbits, AKO7 and AKO8, was purified where indicated by DEAE Affi-Gel Blue (Bio-Rad) affinity chromatography according to the manufacturer's instructions.

In vitro-translated protein mixing

In vitro-translated [^{35}S]methionine-labeled protein was produced in rabbit reticulocyte lysate (Promega) from *in vitro*-transcribed RNA. The truncated E1A product used in

Fig. 1 was transcribed from pZ150.6 (a carboxy-terminal deletion clone of pZ150 consisting of the first 514 nucleotides of the E1A cDNA sequence) and digested with *Bgl*I, resulting in only the first 120 amino acids of E1A being translated. pZ150.6 was linearized and used for *in vitro* transcription with T7 RNA polymerase (Promega). The cRNA was then added to rabbit reticulocyte lysate for *in vitro* translation.

Western blot analyses

Mouse 3T6 cells were infected with wild-type or mutant virus at a multiplicity of 5 PFU/cell. At the indicated times postinfection (p.i.) 2.5×10^6 cells were harvested and lysed in 40 μl of cracking buffer (0.01 *M* $NaPO_4$, pH 7.2, 1% β-mercaptoethanol, 1% SDS, 6 *M* urea) at 37° for 30–180 min. An equal volume of 2× Laemmli gel sample buffer (LGSB, 0.125 *M* Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue) was added to the cell extract and boiled 15 min. Twenty microliters was electrophoresed on a denaturing 10% polyacrylamide gel. MAV-1 E1A protein was detected by Western analysis using the Amersham ECL Western detection kit with a 1:2000 dilution of the MAV-1 E1A polyclonal antiserum, AKO7-147, described above.

Immunoprecipitation of E1A from infected cells

Mouse L929 cells grown on 60-mm plates (2×10^6 cells/plate) were infected with wild-type MAV-1 at a multiplicity of 5 PFU/cell for 48 hr. Cytosine arabinoside (20 μg/ml) was added to the media 9 hr p.i. and was replenished every 8–12 hr. Prior to labeling, cells were washed three times with 0.15 *M* NaCl or 1× PBS and resuspended in media lacking phosphate or methionine and supplemented with 2% dialyzed newborn calf serum. After 3 hr, 1 mCi $^{32}PO_4$ or 200 μCi [^{35}S]methionine was added to the cells in 2 ml of media. Cells were maintained in label for 3 hr, washed once with 1× PBS, then lysed in 1 ml of E1A lysis buffer (250 m*M* NaCl, 50 m*M* Tris, pH 7.4, 0.1% Nonidet-P40, supplemented with 0.1 mg/ml aprotinin) for 30 min on ice. Lysates were precleared with 20 μl of preimmune serum and 200 μl of fixed *Staphylococcus aureus* for 2 hr. Two microliters of preimmune (AKO7-0) or α-E1A antiserum (AKO7-147) was added to $\frac{1}{4}$ of the precleared supernatant from one plate and rotated for 1 hr at 4°, followed by the addition of 200 μl of fixed *S. aureus* and further rotation for 30 min at 4°. Immune complexes were purified by washing the *S. aureus* three times with 1 ml E1A lysis buffer then once with 1 ml 50 m*M* Tris, pH 6.8. Forty microliters of 2× LGSB was added to the purified complexes and boiled for 5 min, and proteins were resolved on SDS–polyacrylamide gels (Laemmli, 1970). Labeled proteins were visualized by fluorography and autoradiography.

In vitro-translated protein/infected cell lysate mixing

Mouse 3T6 cells were infected as described for Western analysis. At 19 and 38 hr p.i., 9×10^6 cells were harvested and lysed in 40 μl of E1A lysis buffer. Ten microliters of infected cell lysate, 20 μl *in vitro*-translated mouse pRb or mouse p107 labeled with [^{35}S]Trans label (ICN), and 220 μl E1A lysis buffer were mixed by rotation at 4° for 1 hr. *In vitro*-translated mouse pRb and mouse p107 for these experiments were obtained from coupled transcription/translation of pCiteRb and pmp107, respectively, using the TNT kit from Promega with [^{35}S]Trans label. Proteins were precipitated with the appropriate antisera (1 μl of purified normal rabbit serum, 1 μl of purified MAV-1 E1A polyclonal antiserum, 25 μl of p107 monoclonal antiserum, or 1 μl of purified mouse pRb polyclonal antiserum) by rotating at 4° for 30 min. Complexes were collected by addition of 100 μl protein A–Sepharose (Pharmacia Biotech; 10% v/v in E1A lysis buffer) and continued rotation for 30 min at 4°. Immune complexes were purified by washing the Sepharose three times with 1 ml E1A lysis buffer and once with 1 ml 50 m*M* Tris, pH 6.8. Forty microliters of 2× LGSB was added to the Sepharose and boiled for 5 min, and proteins were resolved on SDS–polyacrylamide gels. Labeled proteins were detected using a phosphorimager (Molecular Dynamics) and quantitated using ImageQuant software (Molecular Dynamics).

SAOS-2 transfection experiments

SAOS-2 cells (7×10^5) on 60-mm plates were transfected as described (Hinds *et al.,* 1992). Transfections included 3.9 μg of the mouse pRb expression plasmid mRb-SVE, plus 3.9 μg MAV-1 E1A or MAV-1 E1A mutant plasmid (pCME1A, pCMV-CR1Δ, pCMV-CR2Δ, pCMV-CR3Δ, or pCMV-TTG) or vector control (pSVE), and 0.4 μg of pBSpacΔp [provided by R. Ivarie, U. of Georgia (de la Luna *et al.,* 1988)], a plasmid encoding puromycin resistance. All transfections included 0.25 μg/ml puromycin and were supplemented with herring sperm DNA to obtain a total of 12 μg DNA/60-mm plate. Flat cell assays and quantitation were done as described (Hinds *et al.,* 1992).

Mobility shift assays

E2F electrophoretic mobility shift assays were performed using nuclear extracts prepared by the modified method of Dignam *et al.* (1983; Abmayr and Workman, 1994). A double-stranded E2F oligonucleotide (66-mer) corresponding to the double site probe of Obert *et al.* (1994) was used, except that it had additional sequence at the 3′ end (GGGATCCACTAGTT), corresponding to the multiple cloning site region of the double site probe when it is prepared by *Eco*RI–*Xba*I digestion from pKS-E2a (kindly provided by P. Hearing, State University of New

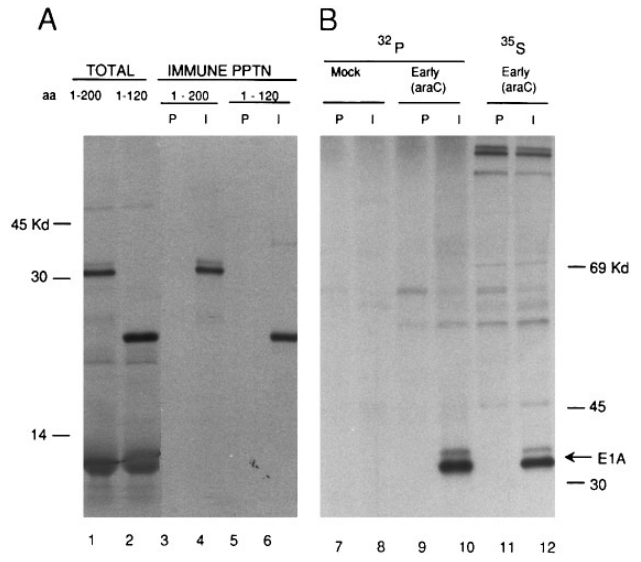

FIG. 1. Immunoprecipitation of E1A proteins. (A) E1A mRNAs were transcribed using T7 RNA polymerase and translated in a rabbit reticulocyte lysate. The [^{35}S]Met-labeled translation products from a full length construct (aa 1–200) and a truncation (aa 1–120) are shown (lanes 1 and 2). The translation products were precipitated with preimmune (P, lanes 3 and 5) or α-E1A-immune (I, lanes 4 and 6) sera and analyzed on a 12% SDS–polyacrylamide gel. (B) MAV-1-infected cells were labeled with $^{32}PO_4$ (lanes 7–10) or [^{35}S]Met (lanes 11 and 12). Cells were mock infected (lanes 7 and 8) or infected for 48 hr in the presence of 20 μg/ml cytosine arabinoside (araC). Antisera were as in A; samples were electrophoresed on a 10% polyacrylamide gel. E1A and positions of molecular weight standards are indicated.

York, Stony Brook). The probe was labeled with [α-^{32}P]-dATP and Klenow polymerase and purified on a 7% acrylamide gel. DNA binding assays (13 μl) contained 2 μg of sonicated salmon sperm DNA, 10 μg of nuclear extract, and 1 μl of monoclonal antibody (where indicated) in 20 m*M* HEPES (*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]), pH 7.5, 100 m*M* KCl, 10% glycerol, 5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.5 m*M* phenylmethylsulfonyl fluoride, 0.1% Nonidet-P40. After a 10-min preincubation at room temperature, 1 μl of probe (50,000 cpm; 17–20 ng) was added and the reactions were incubated for 45 min at room temperature. The reactions were electrophoresed on 4% acrylamide gels at 4°. The control mouse monoclonal antibody, 13D210, was obtained from B. Stillman (Cold Spring Harbor, NY), and was prepared against hAd5 E1B 55K protein.

RESULTS

The predicted MAV-1 E1A protein is made in infected mouse cells

A polyclonal antiserum was produced against aa 27–200 of MAV-1 E1A and was tested for specificity against *in vitro*-transcribed and -translated E1A proteins. Both a full length and a truncated E1A translation product were precipitated with this antiserum but not with the preimmune serum (Fig. 1, lanes 1–6). Mock-infected or wild-type-infected mouse L929 cells were treated with cytosine arabinoside, an inhibitor of DNA replication, to accumulate early proteins (Gaynor *et al.,* 1982). The cells were labeled with $^{32}PO_4$ or [^{35}S]methionine. Lysates were prepared and precipitated with preimmune (Fig. 1, lanes 7, 9, and 11) or α-E1A antiserum (Fig. 1, lanes 8, 10, and 12). A viral-specific band migrating slightly slower than 30 kDa was precipitated from infected cells with the α-E1A antiserum. The 30-kDa size was higher than the predicted molecular weight of 22 kDa for MAV-1 E1A. hAd E1A proteins also migrate more slowly than their predicted molecular weight in SDS–polyacrylamide gels (Yee *et al.,* 1983; Harlow *et al.,* 1985). hAd E1A proteins are phosphorylated although the significance is unknown (Gaynor *et al.,* 1982; Yee *et al.,* 1983; Yee and Branton, 1985a). In the hAds, mutations of phosphorylation sites have not altered the abilities of the E1A proteins to function in the *in vitro* or infected cell culture systems tested (Richter *et al.,* 1988; Dumont *et al.,* 1989). Evidence for phosphorylation of MAV-1 E1A is seen in the immunoprecipitation of ^{32}P-labeled E1A (Fig. 1, lane 10).

In vitro mixing experiments demonstrate an association between E1A and pRb that is dependent on CR2

The presence of the pRb binding motif, D-L-X-C-X-E, in CR2 of MAV-1 E1A prompted an investigation of a potential interaction between MAV-1 E1A and mouse cellular pRb. Using either α-E1A or α-pRb antiserum, coimmunoprecipitation of MAV-1 E1A and mouse pRb was observed using *in vitro*-transcribed and -translated products made from cloned cDNAs of MAV-1 E1A and mouse pRb (data not shown). To determine whether specific regions of MAV-1 E1A are required for the *in vitro* interaction of MAV-1 E1A and mouse pRb, we used MAV-1 E1A cDNAs with deletions in one of the three conserved regions or cDNAs that encoded E1A proteins truncated at the carboxy or amino terminus to produce mutant E1A proteins (Fig. 2). Coimmunoprecipitation of mouse pRb was observed with the CR1 and CR3 deletion mutants as well as with the N-termΔ1 and C-termΔ2 mutants (data not shown). No coimmunoprecipitation of mouse pRb was observed with the CR2 deletion mutant and very little was seen with the N-termΔ2 mutant (data not shown).

Association of pRb and p107 with MAV-1 E1A in infected cell lysates

To examine the sequence requirements for the pRb-E1A association during an infection we used MAV-1 viral mutants. The E1A CR1, CR2, and CR3 deletions used for the *in vitro* experiments were transferred into the MAV-1 genome to produce viral mutants. Mouse 3T6 fibroblasts were infected with each of the mutants and the steady-state E1A protein expression levels were examined by

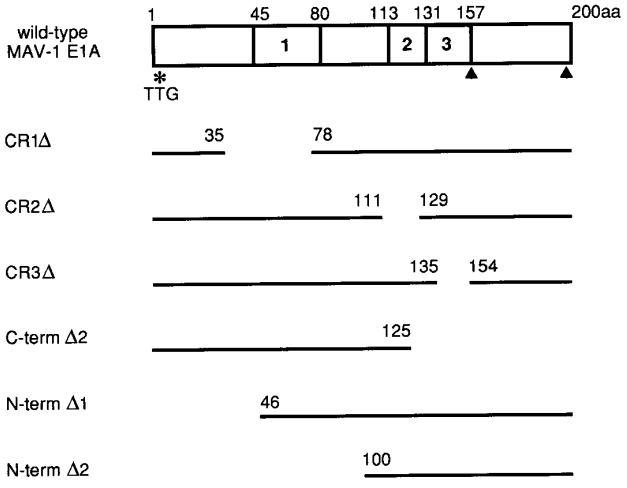

FIG. 2. Schematic diagram of the MAV-1 E1A protein and deletion mutants. The three conserved regions of the protein are contained within the first exon and are depicted as open, numbered boxes with the amino acid numbers indicated above at the boundaries of each region. Splice junctions are identified by the arrows below the diagram at aa 157 and 198. The amino acids flanking the deletion junctions in the mutant E1A proteins are indicated (CR1Δ, CR2Δ, CR3Δ, C-termΔ2, N-termΔ1, N-termΔ2). The TTG mutant (*) consists of a single nucleotide change from an ATG to TTG at the initiator methionine.

Western analysis (Fig. 3). E1A expression was detectable from infected cells at levels comparable to wild type from each of the mutants except the TTG translational initiator mutant, pmE109, from which no E1A expression was detectable. Wild-type E1A protein migrated slightly slower than the 30-kDa marker (Fig. 3, lanes 11 and 12). E1A expressed from dlE102 (CR2Δ) and dlE106 (CR3Δ) migrated faster than wild-type E1A as expected for proteins with deletions of 19 and 20 aa, respectively (Fig. 3, lanes 3, 4, 7, and 8). Despite a 43-amino-acid deletion, confirmed by DNA sequencing, the CR1Δ mutant protein expressed from dlE105 migrated more slowly than the wild-type E1A protein (Fig. 3, lanes 5 and 6). Similar anomalous migration was observed from the *in vitro*-translated CR1 deletion cDNA (data not shown). The reason for this is unknown, but a hAd5 E1A protein mutant with a deletion in CR1 (aa 70–81) also migrates anomalously (Egan *et al.,* 1988).

We were unable to detect pRb from mouse cells in culture. Therefore we examined whether the same regions that are required for an association between *in vitro*-translated E1A and pRb products were required for the association of proteins from infected cells. Mouse 3T6 fibroblasts were infected with wild-type MAV-1 or one of the deletion mutant viruses. Extracts from the infected cells were prepared and mixed with radioactively labeled *in vitro*-translated pRb followed by immunoprecipitation with α-E1A antiserum (Fig. 4). Exogenously added pRb was coimmunoprecipitated from wild-type infected cells at both early and late times p.i. (Fig. 4, lanes 5 and 8). Both dlE105 (CR1Δ)- and dlE106 (CR3Δ)-infected cells showed coimmunoprecipitation of pRb and E1A, although to lower levels, 4% and 55% of wild-type, respectively (Fig. 4, lanes 10, 12, 18, 20). The low proportion of pRb coimmunoprecipitated from dlE105-infected cells compared to wild-type-infected cells may reflect a cooperative role for E1A CR1 in pRb association with E1A. pRb coimmunoprecipitation did not occur with mock-,

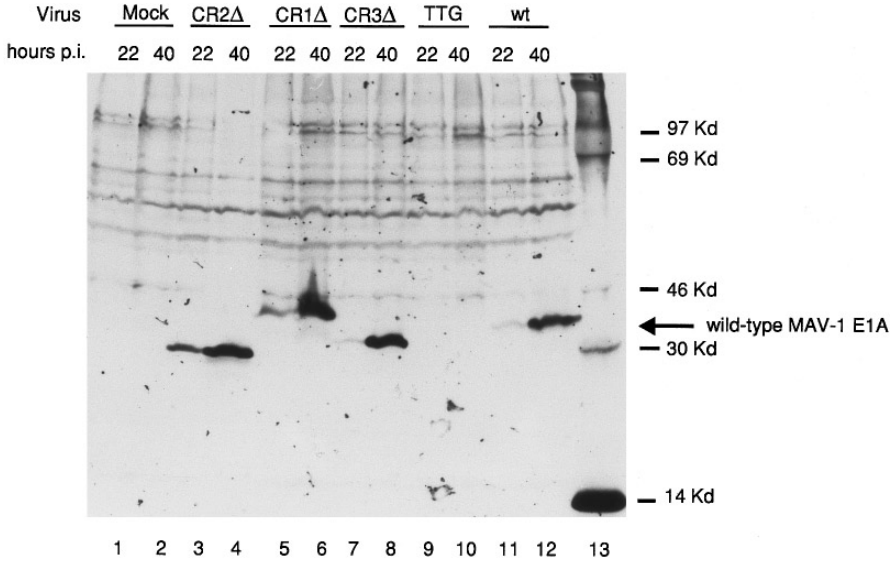

FIG. 3. E1A protein expression from infected mouse 3T6 fibroblasts. Mouse 3T6 cells were infected with wild-type (wt) or mutant MAV-1 at a multiplicity of 5 PFU/cell. Cells were mock infected (lanes 1, 2) or infected for 22 and 40 hr with wt MAV-1 (lanes 11, 12) or one of the following deletion mutants: dlE102 (CR2Δ, lanes 3 and 4), dlE105 (CR1Δ, lanes 5 and 6), dlE106 (CR3Δ, lanes 7 and 8), pmE109 (TTG, lanes 9 and 10). E1A proteins were electrophoresed on a 10% SDS–polyacrylamide gel, blotted to a membrane, and detected by Western analysis with purified α-E1A antiserum, AKO7-147. Wild-type MAV-1 E1A protein, indicated by the arrow on the right, migrates slightly slower than the 30-kDa molecular weight marker.

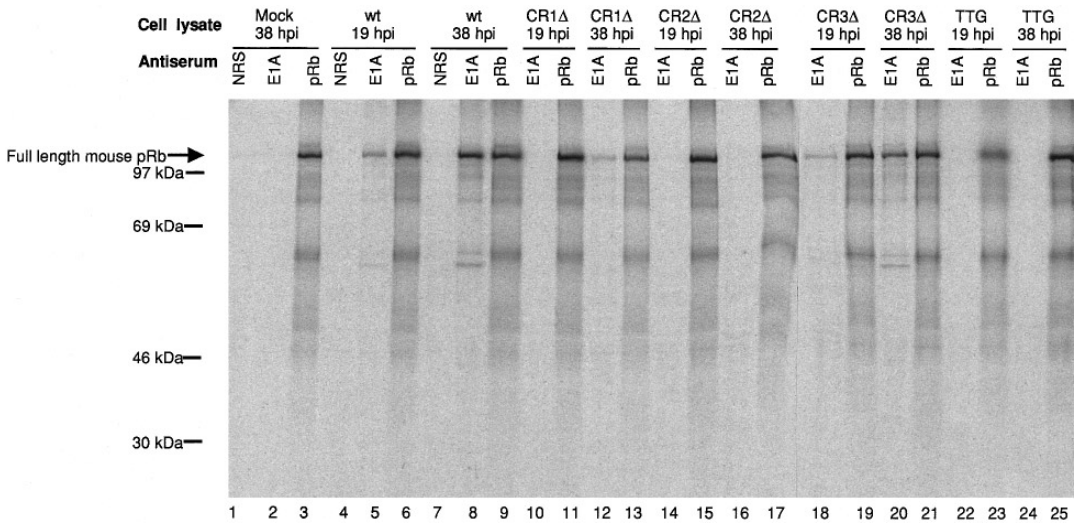

FIG. 4. Coimmunoprecipitation of *in vitro*-translated mouse pRb from infected mouse cell lysates. Mouse 3T6 cells were mock infected (lanes 1–3) or infected with wild-type (wt) or mutant MAV-1, harvested at 19 and 38 hr p.i. (hpi), and lysed in E1A lysis buffer. Equal portions of infected cell lysates were mixed with [^{35}S]Trans-labeled, *in vitro*-translated mouse pRb, and immunoprecipitation was performed with normal rabbit serum (NRS), α-E1A antiserum, or α-mouse pRb antiserum. Immunoprecipitated complexes were resolved on two 12% polyacrylamide gels (lanes 1–17 and 18–25). Positions of full length *in vitro* mouse pRb translation product and the molecular weight markers are indicated. The full length pRb *in vitro* translation product coimmunoprecipitated by the α-E1A antiserum was quantitated by phosphorimetry. Percentages referred to in the text represent the mean value from four independent experiments, where the quantity of pRb coimmunoprecipitated with the E1A protein from cells infected for 38 hr with wt MAV-1, using α-E1A antiserum, was defined as 100%. Amounts of pRb coimmunoprecipitated with the E1A protein from MAV-1 E1A mutant-infected cell lysates are given as the percentage of wt E1A coimmunoprecipitation. CR1Δ, dlE105; CR2Δ, dlE102; CR3Δ, dlE106; TTG, pmE109.

dlE102 (CR2Δ)-, or pmE109 (TTG)-infected cells at either time p.i. (Fig. 4, lanes 2, 14, 16, 22, 24). The results from these experiments are consistent with those of *in vitro* mixing experiments (data not shown).

p107, a cellular protein related to pRb, associates with hAd E1A (Yee and Branton, 1985b; Harlow *et al.,* 1986). The N-terminal portion of CR2 of hAd E1A is required for binding pRb and p107 (Whyte *et al.,* 1989; Dyson *et al.,* 1992b). Exogenously added *in vitro*-translated mouse p107 was coimmunoprecipitated with E1A protein from wild-type virus-infected cells using α-E1A antiserum (Fig. 5, lanes 5 and 8). The ability of MAV-1 E1A mutant proteins to associate with p107 paralleled their ability to bind pRb. A reduced amount of p107 was coimmunoprecipitated with E1A from both dlE105 (CR1Δ)- and dlE106 (CR3Δ)-infected cells (6 and 83% of wild-type, respectively, the mean of three experiments) compared to wild-type infected cells (Fig. 5, lanes 10, 12 and 18, 20). Mouse p107 was not coimmunoprecipitated from mock-, dlE102 (CR2Δ)-, or pmE109 (TTG)-infected cells (Fig. 5, lanes 2, 14, 16, 22, and 24).

MAV-1 E1A and pRb effects on SAOS-2 cells

The *in vitro* coimmunoprecipitation experiments established the existence of a biochemical interaction between MAV-1 E1A and mouse pRb. To demonstrate the functional importance of this interaction, we assayed the ability of MAV-1 E1A to inactivate the function of pRb expressed from a plasmid in SAOS-2 cells, which lack a full length functional pRb. When human pRb is introduced into SAOS-2 cells and expressed, the cells cease to divide and become greatly enlarged due to the ability of pRb to block progression from the G0/G1 phase of the cell cycle (Huang *et al.,* 1988; Goodrich *et al.,* 1991; Mittnacht *et al.,* 1991; Templeton *et al.,* 1991; Hinds *et al.,* 1992). However, coexpression of hAd E1A with pRb in SAOS-2 cells reduces or abolishes the arrested growth phenotype (Hinds *et al.,* 1992). This reversal of growth arrest has been proposed to be due to the ability of hAd E1A to effectively inactivate pRb by associating with it. Similarly, when pRb and antisera specific for pRb were cointroduced into SAOS-2 cells, the arrested growth phenotype was reduced or abrogated (Goodrich *et al.,* 1991). SAOS-2 cells cotransfected with mouse pRb, a control vector plasmid, and a puromycin-resistance plasmid resulted in the characteristic flat, greatly enlarged cell phenotype after 3 days of selection in puromycin that persisted until at least 12 days after selection, when the cells were counted (data not shown). Cotransfection with mouse pRb and wild-type MAV-1 E1A plasmids resulted in a significantly reduced number of enlarged cells. Only 4% of the control number of enlarged cells (resulting from cotransfection of pRb and vector control) was observed when E1A was cotransfected with mouse pRb (Fig. 6). This suggests that mouse pRb is inactivated in the presence of wild-type MAV-1 E1A. To determine which regions of E1A are required for this inactivation of the

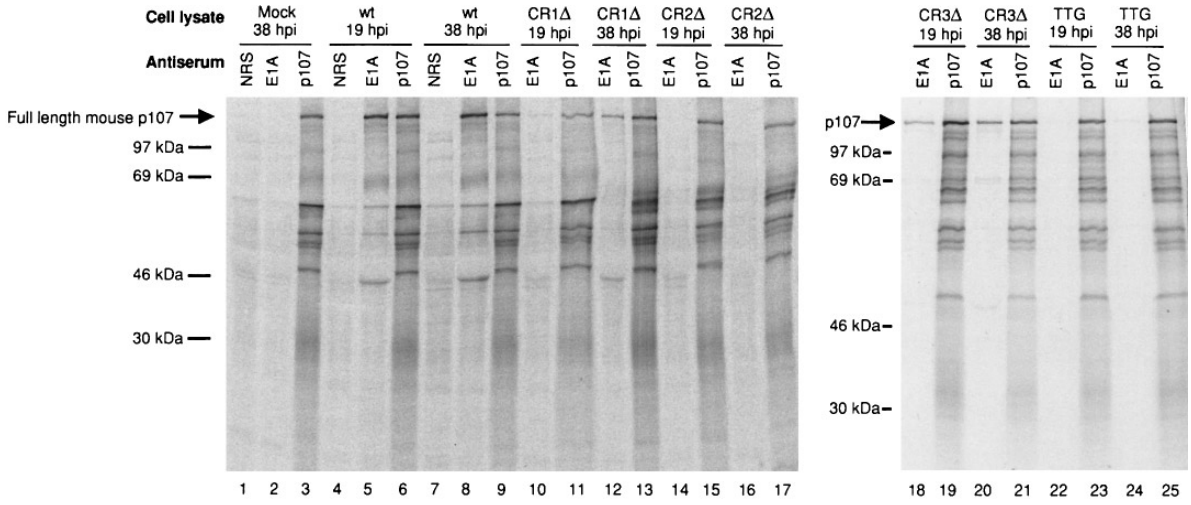

FIG. 5. Coimmunoprecipitation of *in vitro*-translated mouse p107 from infected mouse cell lysates. Infected cell extracts were prepared as in Fig. 4 and equal portions were mixed with [^{35}S]Trans-labeled, *in vitro*-translated mouse p107. Immunoprecipitation was performed with normal rabbit serum (NRS), α-E1A antiserum, or α-p107 antiserum. Immunoprecipitated complexes were resolved on two 12% polyacrylamide gels (lanes 1–17 and 18–25). Positions of full length *in vitro* mouse p107 translation product and the molecular weight markers are indicated. The full length mouse p107 *in vitro* translation product coimmunoprecipitated with wild-type or mutant E1A proteins by the α-E1A antiserum was quantitated as in Fig. 4. Percentages referred to in the text represent the quantitation from three independent experiments. Virus designations are as in Fig. 4.

mouse pRb, various E1A deletion mutant expression plasmids were cotransfected into SAOS-2 cells with the mouse pRb plasmid. Flat, enlarged, nondividing cells were counted for each mutant and compared to the number of enlarged cells produced from pRb alone (Fig. 6). None of the E1A constructs induced the flat cell morphology when transfected in the absence of mouse pRb (data not shown), nor were they toxic to the cells, because patches of dividing cells were observed in transfections with each construct. Unlike cotransfection of the wild-type E1A plasmid, cotransfection of the initiator mutant E1A plasmid (pCMV-TTG) and pRb did not result in a reduced number of flat cells relative to cotransfection of the vector control plasmid and pRb. In fact, the number of flat cells produced by cotransfection of pCMV-TTG was almost double the number resulting from cotransfection of pRb and the control vector. Transfection of SAOS-2 cells with pCMV-TTG in the absence of pRb did not result in flat cells (data not shown) so the increase in the number of flat, enlarged cells in the cotransfection with pRb is not due to a direct activity of the mutant E1A. When the various E1A deletion mutant plasmids were cotransfected with pRb, fewer flat cells resulted than with pRb and vector control, but the reductions were not as large as those seen with wild-type E1A. The coexpression of CR3Δ E1A with pRb reduced the number of flat cells to 11% of the control, whereas the expression of the CR1Δ and CR2Δ mutant E1As resulted in more flat, enlarged cells (34 and 17%, respectively). This indicates that the CR1Δ and CR2Δ E1A proteins were less effective at reversing the effect of mouse pRb in this assay than CR3Δ or wild-type E1A.

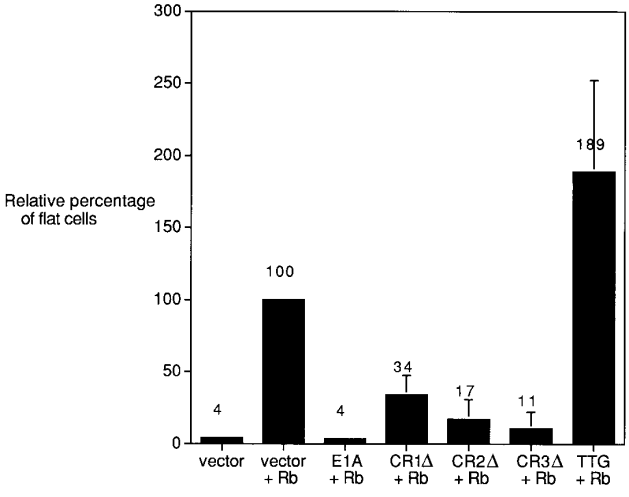

FIG. 6. Quantitation of flat, enlarged cell production by cotransfection of SAOS-2 cells with mouse pRb and MAV-1 E1A. SAOS-2 cells were cotransfected with three plasmids: (1) the mouse pRb expression plasmid, mRb-SVE; (2) either a control vector, pSVE, or a MAV-1 E1A expression plasmid; and (3) a puromycin-resistance plasmid, pBSpacΔp. After 12 days of selection in puromycin, the flat, enlarged cells were counted. The fraction of total cells transfected with pRb and the control vector that were enlarged was set at 100% for each experiment. The numbers of flat, enlarged cells counted for each sample are reported as a percentage relative to pRb and the control vector. Quantitation represents the mean from three independent experiments.

p107–E2F complexes in MAV-1-infected cells

p107 is present at low or undetectable levels in the mouse cells we have used for MAV-1 infection, including the L929, 3T6, and SP2/0 cell lines, and primary embryo fibroblasts (Smith and Spindler, unpublished). Therefore we have not been able to detect a p107–MAV-1 E1A complex by coimmunoprecipitation of cellular p107 and

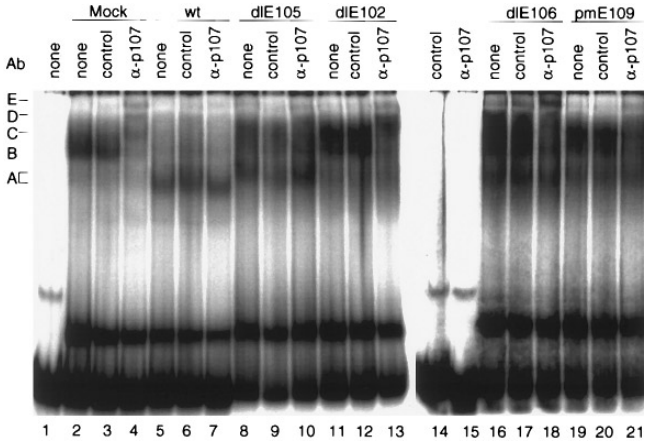

FIG. 7. E2F mobility shift assays. An E2F double-site probe was labeled with ^{32}P and incubated with no cell extract (lanes 1, 14, 15) or mock- or MAV-1 wild-type- or mutant-virus-infected 3T6 cell extracts as indicated (lanes 2–13 and 16–21), and complexes were resolved on two 4% polyacrylamide gels (lanes 1–13 and 14–21). Reactions contained no antibodies ("none") or control (13D210) or α-p107 (SD-9) antibodies as indicated above the lanes. The letters A–E on the left refer to complexes discussed in the text. Ab, antibody.

E1A in infected cells. Mouse p107 associates with the transcription factor E2F and the formation of this complex can be measured in mobility shift assays using an E2F-specific DNA probe (Corbeil *et al.,* 1995). Using a mobility shift assay, we examined whether we could detect an E2F–DNA binding complex in mock-infected cells that contained mouse p107 and that would be absent or reduced in amount in wild-type MAV-1-infected cells (Fig. 7). A ^{32}P-labeled DNA oligonucleotide containing two E2F sites was used in these experiments (Obert *et al.,* 1994). Cell extracts were prepared from mock-infected 3T6 cells or from 3T6 cells infected for 20 hr. The probe showed no specific high-molecular-weight complexes in the absence of cell extract, and was not affected by the presence of control or α-p107 monoclonal antibodies (Fig. 7, lanes 1, 14, 15). Mock-infected extracts produced a complex (band B, Fig. 7, lane 2) which shifted in the presence of α-p107 monoclonal antibodies (bands C, D, and E, lane 4) but not with control antibodies (lane 3). A faster migrating complex was seen in wt-virus-infected extracts (band A, lane 5). Because this complex was unaffected by the presence of α-p107 monoclonal antibodies (lane 7), we conclude that it does not contain p107. This complex was also seen, and also not shifted by the presence of α-p107 monoclonal antibodies, in CR1Δ and CR3Δ mutant-infected extracts (band A, lanes 8–10 and 16–18). The slightly faster mobility of the A complex in wt-infected cells compared to dlE105 and dlE106 was not reproduced in three other experiments (data not shown). For the CR1Δ and CR3Δ mutant extracts, the presence of B/C complexes (similar to those seen in mock) and appearance of additional complexes (D, E) upon addition of α-p107 antibody varied in multiple experiments (lanes 8–10, 16–18, and data not shown). In contrast to the results with wt virus, extracts from cells infected with the CR2Δ or the TTG mutant had little or none of the infected-cell specific complex A, but had complexes similar to the mock complex (bands B and C, lanes 11, 12, 19, and 20). The amount of B/C complexes seen in the CR2Δ and TTG mutant extracts decreased in the presence of α-p107 monoclonal antibodies, with the accompanying appearance of new complexes D or E (lanes 13 and 21). We conclude that E2F complexes that contained p107 formed in the mutant-infected extracts. We suggest that the appearance of these p107-containing complexes was due to the absence of MAV-1 E1A capable of binding p107.

DISCUSSION

Human adenovirus studies have identified interactions that occur between E1A and host cell proteins, most of which are involved in cell cycle regulation. Specific regions of E1A required for these interactions have been defined through extensive mutational analysis. pRb, p107, and p130 require CR2 for efficient association with E1A (Egan *et al.,* 1988; Jelsma *et al.,* 1989; Whyte *et al.,* 1989; Giordano *et al.,* 1991; Dyson *et al.,* 1992a; Barbeau *et al.,* 1993, 1994; Li *et al.,* 1993; Mayo *et al.,* 1993). Deletion of CR1 significantly reduces associations with pRb and p130 but only slightly reduces the amount of p107 bound by E1A (Egan *et al.,* 1988; Barbeau *et al.,* 1993, 1994). CR1 and CR2 appear to play a significant role in both cellular protein binding and a number of E1A-associated functions, suggesting a possible correlation between the two.

pRb, p107, and p130 are involved in the regulation of cell proliferation of quiescent cells. pRb (p105) is the product of the retinoblastoma susceptibility gene and is a tumor suppressor protein. Hypophosphorylated pRb forms a complex with and inhibits the activity of certain E2F (E2F-1,2,3) transcription factors during the G1 phase of the cell cycle. p107 and p130, pRb-related phosphoproteins, also form complexes with specific E2F transcription factors and cyclin A (p60^{cycA}) and E in a cell cycle-dependent manner during the G1 and S phases or during the G0 and G1 phases, respectively (Cao *et al.,* 1992; Devoto *et al.,* 1992; Lees *et al.,* 1992; Shirodkar *et al.,* 1992; Cobrinik *et al.,* 1993). These phosphoprotein/E2F complexes are disrupted when hAd E1A binds to either pRb or p107 (Bagchi *et al.,* 1990; Bandara and La Thangue, 1991; Chellappan *et al.,* 1991; Mudryj *et al.,* 1991), enabling the E2F transcription factors to activate promoters of both viral and cellular genes containing E2F sites, including many S-phase-specific genes. Some hAd E1A CR2 deletion or point mutants that fail to bind pRb efficiently yet maintain association with p107 show reduced levels of *in vitro* E2F activity, presumably due to a lack of free and active E2F (Corbeil and Branton, 1994).

There are other CR2 point mutants that are unable to bind pRb yet bind p107 or p130 and activate E2F, suggesting that E2F activation may also result from its release from p107 or p130 (Corbeil and Branton, 1994). Release of E2F from complexes with these cellular proteins triggers entry of the cell into S phase, allowing both cellular and viral DNA synthesis to occur. Thus adenoviruses manipulate the cells they infect through viral and host protein interactions to create a favorable environment for their replication.

In these studies MAV-1 E1A associated with at least two of the cellular proteins found to associate with hAd E1A. Experiments with *in vitro*-translated E1A and mouse proteins demonstrated an E1A association with both mouse pRb and p107 (data not shown). Results from the *in vitro* mixing experiments with mouse pRb and mutant E1As showed essentially no E1A–pRb association with either a CR2 deletion or an N-terminal truncation of the first 100 amino acids (thereby lacking CR1) (data not shown). However, the CR1 deletion and the CR3 deletion mutant had detectable levels of pRb association. Therefore CR2 and a portion of the N-terminus of E1A appear to be required for association to occur between the two *in vitro*-translated proteins.

Attempts to detect the E1A–pRb interactions directly in infected cells by coimmunoprecipitation were unsuccessful (Smith and Spindler, unpublished), possibly for the following reasons. pRb was not produced at detectable levels in the mouse L929 and 3T6 cells that we used for infection by MAV-1, as measured by immunoprecipitation of labeled infected cells or by Western blot analysis (Smith and Spindler, unpublished). One cell line, SP2/O (mouse splenocytes), produced detectable levels of both mouse pRb and mouse p107, but it was not productively infected by MAV-1. In addition, a protein of approximately 100 kDa is produced in infected cells which we were unable to preclear from extracts using a variety of sera. Thus the presence of this protein in all immunoprecipitations from infected cells obscured any pRb that would have been present in a complex with E1A. Because of the difficulties of demonstrating an interaction in radiolabeled infected cells by coimmunoprecipitation, we used two other experimental approaches to confirm the *in vitro* results.

Use of MAV-1 mutant viruses with deletions in the E1A region demonstrated that the same E1A regions that were required *in vitro* were required for association with both mouse pRb and mouse p107 in coimmunoprecipitation experiments using infected cell extracts (Figs. 4 and 5). Both pRb and p107 were coimmunoprecipitated from dlE106-infected cells which express a CR3Δ mutant E1A protein (55 and 83% of wild-type, respectively). Neither pRb nor p107 was coimmunoprecipitated from mock or pmE109 (TTG)-infected cells which did not have E1A in the lysates. Little or no pRb was coimmunoprecipitated from dlE105 (CR1Δ)- or dlE102 (CR2Δ)-infected cells (4 or 0% of wild-type, respectively). Similar results were obtained for mouse p107 in dlE105- and dlE102-infected cells. Thus both CR1 and CR2 are needed for efficient binding of pRb and p107 by MAV-1 E1A during an infection in cell culture. These results are consistent with hAd studies.

The biochemical interactions reported here were corroborated by functional assays for the interaction between MAV-1 E1A and mouse pRb or p107. SAOS-2 cells, which lack a functional pRb, were used to assay the effects of MAV-1 E1A on mouse pRb. Introduction of mouse pRb into SAOS-2 cells induced the same phenotype of arrested growth as does introduction of human pRb. This was expected since mouse pRb and human pRb protein sequences are 91% identical (Bernards *et al.,* 1989). Our experiments demonstrated that MAV-1 E1A reversed the effect of mouse pRb (similar to the effect of hAd E1A on human pRb) when cointroduced into SAOS-2 cells. This E1A effect was dependent on the presence of CR1 and CR2, the two regions found to be most important for interacting with mouse pRb in infected cells. The CR3Δ E1A had only a slightly reduced ability to alter the phenotype induced by pRb in SAOS-2 cells compared to wild-type E1A. This correlates with its slightly reduced ability to bind pRb compared to wild-type E1A (Fig. 4). The reasons for the increased number of flat cells seen with the TTG mutant E1A are not known. Even if some E1A was translated from the TTG, it would not explain the increase in the number of flat cells produced, since the coexpression of wild-type E1A with pRb significantly reduced the number of flat cells. In summary, the SAOS-2 cell experiments demonstrated that MAV-1 E1A protein expression in SAOS-2 cells altered the pRb-induced phenotype, suggesting that the interaction between E1A and pRb inactivated the pRb. Inactivation of pRb is hypothesized to deregulate the cell cycle of the infected cell, creating a more permissive environment for viral replication (Whyte *et al.,* 1988).

The biochemical interaction between E1A and p107 was corroborated in mobility shift experiments using infected cell extracts. No p107-containing E2F complexes were identified in wt-infected cells, whereas they were readily seen in mock-infected cells (Fig. 7 and data not shown). We infer that this is because in infected cells p107 is complexed with E1A. In the CR2Δ and TTG mutants that were unable to produce E1A that could associate with p107 (Fig. 5), p107–E2F complexes were seen in mobility shift experiments (Fig. 7). This is consistent with some p107 not complexing with E1A and therefore being available for binding to E2F. This result provides genetic evidence for the assertion that wt MAV-1 E1A associates with p107 and that CR2 is essential for this association.

The requirement for E1A CR1 and CR3 for binding to p107 was not resolved in these experiments. CR1 and CR3 were less important than CR2, since p107 was found

to associate with CR1Δ and CR3Δ mutants. The coimmunoprecipitation assay indicated that CR1 was of more importance for E1A binding to p107 than was CR3. In the mobility shift experiments shown in Fig. 7, deletion of CR3 seemed to reduce E1A binding to p107 more than did deletion of CR1. However, in additional mobility shift experiments, this relative importance of CR1 and CR3 was reversed (data not shown). Thus the relative and absolute requirements for CR1 and CR3 could not be ascertained from the data we have obtained, although it is clear that deleting CR1 and CR3 had significantly less effect on E1A binding to p107 than did deleting E1A CR2 or E1A expression entirely.

Preliminary studies have shown that MAV-1 infection of serum-deprived growth-arrested mouse 3T6 cells reversed the G0/G1 block, allowing the cells to reenter the cell cycle (Smith and Spindler, unpublished). Further experiments will determine what effects the different E1A conserved region deletions will have on growth-arrested cells.

Our ultimate goal is to determine the function of E1A in pathogenesis during MAV-1 infection in mice. Specific effects of E1A viral mutants identified in cell culture infections will enable us to correlate functions of E1A with particular aspects of pathogenesis. Loss of the ability to associate with and inactivate cellular proteins during an infection may have drastic affects in the animal.

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REFERENCES

Abmayr, S. M., and Workman, J. L. (1994). Preparation of nuclear and cytoplasmic extracts from mammalian cells. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds.), pp. 12.1.1–12.1.9. Wiley, New York.

Bagchi, S., Raychaudhuri, P., and Nevins, J. R. (1990). Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: A novel mechanism for E1A *trans*-activation. *Cell* 62, 659–669.

Ball, A. O., Beard, C. W., Redick, S. D., and Spindler, K. R. (1989). Genome organization of mouse adenovirus type 1 early region 1: A novel transcription map. *Virology* 170, 523–536.

Ball, A. O., Williams, M. E., and Spindler, K. R. (1988). Identification of mouse adenovirus type 1 early region 1: DNA sequence and a conserved transactivating function. *J. Virol.* 62, 3947–3957.

Bandara, L. R., and La Thangue, N. B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* 351, 494–497.

Barbeau, D., Charbonneau, R., Whalen, S. G., Bayley, S. T., and Branton, P. E. (1994). Functional interactions within adenovirus E1A protein complexes. *Oncogene* 9, 359–373.

Barbeau, D., Marcellus, R. C., Bacchetti, S., Bayley, S. T., and Branton, P. E. (1993). Quantitative analysis of regions of adenovirus E1A products involved in interactions with cellular proteins. *Biochem. Cell Biol.* 70, 1123–1134.

Beard, C. W., and Spindler, K. R. (1996). Analysis of early region 3 mutants of mouse adenovirus type 1. *J. Virol.,* 70, 5867–5874.

Beard, C. W., Ball, A. O., Wooley, E. H., and Spindler, K. R. (1990). Transcription mapping of mouse adenovirus type 1 early region 3. *Virology* 175, 81–90.

Berk, A. J., Lee, F., Harrison, T., Williams, J., and Sharp, P. A. (1979). Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. *Cell* 17, 935–944.

Bernards, R., Schackleford, G. M., Gerber, M. R., Horowitz, J. M., Friend, S. H., Schartl, M., Bogenmann, E., Rapaport, J. M., McGee, T., Dryja, T. P., and Weinberg, R. A. (1989). Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. *Proc. Natl. Acad. Sci. USA* 86, 6474–6478.

Borelli, E., Hen, R., and Chambon, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* 312, 608–612.

Boulanger, P. A., and Blair, G. E. (1991). Expression and interactions of human adenovirus oncoproteins. *Biochem. J.* 275, 281–299.

Braithwaite, A. W., Cheetham, B. F., Li, P., Parish, C. R., Waldron-Stevens, L. K., and Bellett, A. J. D. (1983). Adenovirus-induced alterations of the cell growth cycle: A requirement for expression of E1A but not of E1B. *J. Virol.* 45, 192–199.

Cao, L., Faha, B., Dembski, M., Tsai, L.-H., Harlow, E., and Dyson, N. (1992). Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature* 355, 176–179.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991). The E2F transcription factor is a cellular target for the RB protein. *Cell* 65, 1053–1062.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7, 2745–2752.

Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T., and Weinberg, R. A. (1993). Cell cycle specific association of E2F with the p130 E1A-binding protein. *Genes Dev.* 7, 2392–2904.

Corbeil, H. B., and Branton, P. E. (1994). Functional importance of complex formation between the retinoblastoma tumor suppressor family and adenovirus E1A proteins as determined by mutational analysis of E1A conserved region 2. *J. Virol.* 68, 6697–6709.

Corbeil, H. G., Whyte, P., and Branton, P. E. (1995). Characterization of transcription factor E2F complexes during muscle and neuronal differentiation. *Oncogene* 11, 909–920.

de la Luna, S., Soria, I., Pulido, D., Ortin, J., and Jimenez, A. (1988). Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. *Gene* 62, 121–126.

Devoto, S. H., Mudryj, M., Pines, J., Hunter, T., and Nevins, J. R. (1992). A cyclin A–protein kinase complex possesses sequence-specific DNA binding activity: $p33^{cdk2}$ is a component of the E2F–cyclin A complex. *Cell* 68, 167–176.

Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11, 1475–1489.

Dumont, D. J., Tremblay, M. L., and Branton, P. E. (1989). Phosphorylation at serine 89 induces a shift in gel mobility but has little effect

on the function of adenovirus type 5 E1A proteins. *J. Virol.* 63, 987–991.

Dunsworth-Browne, M., Schell, R. E., and Berk, A. J. (1980). Adenovirus terminal protein protects single stranded DNA from digestion by a cellular exonuclease. *Nucleic Acids Res.* 8, 543–554.

Dyson, N., Dembski, M., Fattaey, A., Ngwu, C., Ewen, M., and Helin, K. (1993). Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB-associated E2F-1. *J. Virol.* 67, 7641–7647.

Dyson, N., Guida, P., McCall, C., and Harlow, E. (1992a). Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. *J. Virol.* 66, 4606–4611.

Dyson, N., Guida, P., Münger, K., and Harlow, E. (1992b). Homologous sequence in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J. Virol.* 66, 6893–6902.

Egan, C., Jelsma, T. N., Howe, J. A., Bayley, S. T., Ferguson, B., and Branton, P. E. (1988). Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Mol. Cell. Biol.* 8, 3955–3959.

Gaynor, R. B., Hillman, D., and Berk, A. J. (1984). Adenovirus early region 1A protein activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. *Proc. Natl. Acad. Sci. USA* 81, 1193–1197.

Gaynor, R. B., Tsukamoto, A., Montell, C., and Berk, A. J. (1982). Enhanced expression of adenovirus transforming proteins. *J. Virol.* 44, 276–285.

Giordano, A., McCall, C., Whyte, P., and Franza, B. R. (1991). Human cyclin A and the retinoblastoma protein interact with similar but distinguishable sequences in the adenovirus E1A gene product. *Oncogene* 6, 481–485.

Goodrich, D. W., Wang, N. P., Qian, Y.-W., Lee, W. Y.-H. P., and Lee, W.-H. (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* 67, 293–302.

Gorman, C., Padmanabhan, R., and Howard, B. H. (1983). High efficiency DNA-mediated transformation of primate cells. *Science* 221, 551–553.

Harlow, E., Franza, B. R., Jr., and Schley, C. (1985). Monoclonal antibodies specific for adenovirus early region 1A proteins: Extensive heterogeneity in early region 1A products. *J. Virol.* 55, 533–546.

Harlow, E., Whyte, P., Franza, B. R. J., and Schley, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* 6, 1579–1589.

Hen, R., Borelli, E., and Chambon, P. (1985). Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1A products. *Science* 230, 1391–1394.

Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 70, 993–1006.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26, 365–369.

Howe, J. A., and Bayley, S. T. (1992). Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. *Virology* 186, 15–24.

Howe, J. A., Mymryk, J. S., Egan, C., Branton, P. E., and Bayley, S. T. (1990). Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. *Proc. Natl. Acad. Sci. USA* 87, 5883–5887.

Hu, Q., Bautista, C., Edwards, G. M., Defeo-Jones, D., Jones, R. E., and Harlow, E. (1991). Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. *Mol. Cell. Biol.* 11, 5792–5799.

Huang, H.-J., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E. Y.-H. P., and Lee, W.-H. (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* 242, 1563–1566.

Jelsma, T. N., Howe, J. A., Mymryk, J. S., Evelegh, C. M., Cunniff, N. F. A., and Bayley, S. T. (1989). Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. *Virology* 171, 120–130.

Jones, N., and Shenk, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 17, 683–689.

Koerner, T. J., Hill, J. E., Myers, A. M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of *trpE*-fusion genes: pATH vectors. *Methods Enzymol.* 194, 477–490.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Lees, E., Faha, B., Dulic, V., Reed, S. I., and Harlow, E. (1992). Cyclin E/cdk2 and cyclinA/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes Dev.* 6, 1874–1885.

Li, Y., Graham, C., Lacy, S., Duncan, A. M. V., and Whyte, P. (1993). The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes Dev.* 7, 2366–2377.

Mayo, X., Grana, X., Baldi, A., Sang, N., Hu, Q., and Giordano, A. (1993). Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. *Oncogene* 8, 2561–2566.

Mittnacht, S., Hinds, P. W., Dowdy, S. F., and Weinberg, R. A. (1991). Modulation of retinoblastoma protein activity during the cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* 56, 197–209.

Moran, B., and Zerler, B. (1988). Interactions between cell growth-regulating domains in the products of the adenovirus E1A oncogene. *Mol. Cell. Biol.* 8, 1756–1764.

Morgenstern, J. P., and Land, H. (1990). A series of mammalian expression vectors and characterization of their expression of a reporter gene in stably and transiently transfected cells. *Nucleic Acids Res.* 18, 1068.

Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J., and Nevins, J. R. (1991). Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. *Cell* 65, 1243–1253.

Nevins, J. R. (1982). Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* 29, 913–919.

Obert, S., O'Connor, R. J., Schmid, S., and Hearing, P. (1994). The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. *Mol. Cell. Biol.* 14, 1333–1346.

Raviprakash, K. S., Grunhaus, A., El Kholy, M. A., and Horwitz, M. S. (1989). The mouse adenovirus type 1 contains an unusual E3 region. *J. Virol.* 63, 5455–5458.

Richter, J. D., Slavicek, J. M., Schneider, J. F., and Jones, N. C. (1988). Heterogeneity of adenovirus type 5 E1A proteins: Multiple serine phosphorylations induce slow-migrating electrophoretic variants but do not affect E1A-induced transcriptional activation or transformation. *J. Virol.* 62, 1948–1955.

Ruley, H. E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* 304, 602–606.

Shenk, T., and Flint, J. (1991). Transcriptional and transforming activities of the adenovirus E1A proteins. *Adv. Cancer Res.* 57, 47–85.

Shimojo, H., and Yamashita, T. (1968). Induction of DNA synthesis by adenoviruses in contact-inhibited hamster cells. *Virology* 36, 422–433.

Shirodkar, S., Ewen, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. (1992). The transcription factor E2F interacts with the retinoblastoma product and a p107–cyclin A complex in a cell cycle-regulated manner. *Cell* 68, 157–166.

Simon, M. C., Kitchener, K., Kao, H.-T., Hickey, E., Weber, L., Voellmy, R., Heintz, N., and Nevins, J. R. (1987). Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. *Mol. Cell. Biol.* 7, 2884–2890.

Spindler, K. R., Eng, C. Y., and Berk, A. J. (1985). An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. *J. Virol.* 53, 742–750.

Stein, R. W., Corrigan, M., Yaciuk, P., Whelan, J., and Moran, E. (1990). Analysis of E1A-mediated growth regulation functions: Binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. *J. Virol.* 64, 4421–4427.

Sterner, J. M., Murata, Y., Kim, H. G., Kennett, S. B., Templeton, D. J., and Horowitz, J. M. (1995). Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in G2/M phases. *J. Biol. Chem.* 270, 9281–9288.

Svensson, C., and Akusjärvi, G. (1984). Adenovirus 2 early region 1A stimulates expression of both viral and cellular genes. *EMBO J.* 3, 789–794.

Templeton, D. J., Park, S.-H., Lanier, L., and Weinberg, R. A. (1991). Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proc. Natl. Acad. Sci. USA* 88, 3033–3037.

Velcich, A., and Ziff, E. (1985). Adenovirus E1A proteins repress transcription from the SV40 early promoter. *Cell* 40, 705–716.

Wang, H.-G. H., Rikitake, Y., Carter, M. C., Yaciuk, P., Abraham, S. E., Zerler, B., and Moran, E. (1993). Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth. *J. Virol.* 67, 476–488.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* 334, 124–129.

Whyte, P., Williamson, N. M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell* 56, 67–75.

Yee, S.-P., and Branton, P. E. (1985a). Analysis of multiple forms of human adenovirus type 5 E1A polypeptides using an antipeptide antiserum specific for the amino terminus. *Virology* 146, 315–322.

Yee, S.-P., and Branton, P. E. (1985b). Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology* 147, 142–153.

Yee, S.-P., Rowe, D. T., Tremblay, M. L., McDermott, M., and Branton, P. E. (1983). Identification of human adenovirus early region 1 products using antisera against synthetic peptides corresponding to the predicted carboxy termini. *J. Virol.* 46, 1033–1013.

Younghusband, H. B., Tyndall, C., and Bellett, A. J. D. (1979). Replication and interaction of virus DNA and cellular DNA in mouse cells infected by a human adenovirus. *J. Gen. Virol.* 45, 455–467.

Zerler, B., Roberts, R. J., Mathews, M. B., and Moran, E. (1987). Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. *Mol. Cell. Biol.* 7, 821–829.

Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.* 7, 1111–1125.